ANTIGENS DERIVED FROM FILAGGRINS AND THEIR USE FOR THE DIAGNOSIS OF RHEUMATOID ARTHRITIS

The present invention relates to new preparations of antigens specifically recognized by autoantibodies specific for rheumatoid arthritis.

Rheumatoid arthritis (hereinafter abbreviated "RA") is the most frequent of the chronic inflammatory rheumatisms. It is an autoimmune disease, and the serum of affected patients contains autoantibodies of which some are specific, and may constitute a marker for this disease, allowing its diagnosis even at early stages. Research studies have therefore been carried out in order to identify antigens recognized by these antibodies, in order to obtain therefrom purified preparations which can be used in conventional immunological diagnostic techniques.

Autoantibodies which are specifically present in patients suffering from RA and which react with a rat esophageal epithelial antigen were described for the first time by B.J.J. Young et al. in Br. Med. J. 2:97-99, (1979). These autoantibodies were at the time called "antikeratin antibodies".

During previous studies, the inventors' team obtained, from human and murine malpighian epithelia, preparations of antigens related to filaggrin and to profilaggrin, which are specifically recognized by the antibodies present in the serum of patients suffering from rheumatoid arthritis, and showed that the "antikeratin antibodies" were in fact anti-filaggrin autoantibodies (hereinafter called "AFA"). Application EP 0,511,116 describes these antigenic preparations and their use for the diagnosis of rheumatoid arthritis.

Filaggrins are a family of proteins which has been identified in various species, inter alia in humans, rats, mice, guinea pigs, at the level of the keratinizing malpighian epithelia [for a review on filaggrins, cf. DALE et al. [The Keratinocyte Handbook, Cambridge University Press, pp 323-350, (1994)]. They

are derived from the dephosphorylation and from the proteolysis of a precursor, profilaggrin, which essentially consists of repeated domains of filaggrin separated by interdomain peptide segments.

The gene encoding profilaggrin is composed of repeating subunits each of which encodes a molecule of filaggrin, which are separated by portions encoding the interdomain peptide segments. All the repeating units encoding each of the human filaggrins have the same length (972 base pairs in humans); however, in humans, large (10-15%) sequence variations are observed from one subunit to another. While the majority are conservative, some of these variations induce changes in amino acids and in some cases changes in the electrical charge on the protein. Thus, human filaggrins form, independently of the post-transcriptional modifications, a heterogeneous population of molecules with a similar size but with different sequences and charges (pHi equal to 8.3 ± 1.1) [GAN et al., Biochem. 29, p. 9432-9440 (1990)].

Profilaggrin is a protein with a high molecular weight (about 400,000 in humans) which is soluble in the presence of high salt or urea concentrations. It has a high content of basic amino acids (arginine and histidine), as well as of glycine, serine and glutamic acid. It is low in nonpolar amino acids and does not contain methionine, cysteine or tryptophan. It is highly phosphorylated on serine residues, which confers on it an isoelectric point close to neutrality.

Profilaggrin is cleaved into filaggrin units during a complex process of maturation involving dephosphorylation, followed by cleavage by proteases at the level of the interdomain segments. This cleavage first generates fragments of intermediate size, and then the functional molecules of filaggrin.

The filaggrins derived from the dephosphorylation and cleavage of profilaggrin are basic proteins whose content of amino acids is similar

to that of the profilaggrins. They participate in the organization of the keratin filaments and undergo gradual maturation during which the basic arginine residues are converted to neutral citrulline residues under the action of peptidylarginine deiminase [HARDING C.R. and SCOTT I.R., J. Mol. Biol. 170, p. 651-673 (1983)]. This causes a reduction in their affinity for the keratins from which they become detached; they are then completely degraded under the action of various proteases.

The properties of filaggrins and profilaggrins have been particularly well studied in rats, in mice and in humans. The size of profilaggrin varies, depending on the species, from 300 to 400 kD and that of the filaggrins from 27 to 64 kD.

The polymorphism observed in humans between the sequences of filaggrin units within the same profilaggrin gene does not appear in rats and mice. The filaggrins exhibit, in addition, a high inter- and intra-specific variability at the level of their sequence. This variability does not however affect their functional properties or their overall amino acid composition, and their biochemical properties. Likewise, the tissue locations of profilaggrin and of filaggrins are identical in the various mammals studied.

Continuing their studies, the inventors observed that the profilaggrin present in the keratohyalin granules of the human epidermis was not, contrary to the filaggrins, recognized by AFAs [SIMON et al. Clin. Exp. Immunol. 100, 90-98 (1995)]. They then tested the reactivity of the AFAs with recombinant filaggrin, and observed that the latter was not recognized either. On the other hand, it had been previously observed that the forms of the human epidermal filaggrins mainly recognized by the AFAs were the acido-neutral forms described by SIMON et al. [J. Clin. Invest., 92, 1387, (1993)] and in application EP 0,511,116. The fact that these acido-neutral forms

correspond to a late stage of maturation of filaggrin made it possible to suppose that all or part of the post-translational modifications occurring up to this stage were involved in the formation of the epitopes recognized by the AFAs.

To verify this hypothesis, the inventors sought to reproduce *in vitro*, using recombinant filaggrin, these post-translational modifications in order to determine the ones which were capable of influencing the antigenicity of filaggrin.

They thus observed that in fact the citrullination of filaggrin was sufficient to generate epitopes recognized by the AFAs. Indeed, they observed, by carrying out the deimination *in vitro* of recombinant filaggrin, that the replacement of at least part of the arginines with citrullines allows an antigen to be obtained which is specifically recognized by the AFAs present in the serum of patients suffering from RA.

The subject of the present invention is an artificial antigen which is specifically recognized by the AFAs present in the serum of patients suffering from RA, characterized in that it consists of a recombinant or synthetic polypeptide comprising all or part of a sequence derived from that of a filaggrin unit or of a related molecule, by replacing at least one arginine residue with a citrulline residue. Preferably, an antigen in accordance with the invention comprises at least 5 consecutive amino acids, and advantageously at least 10 consecutive amino acids, including at least one citrulline, of said sequence.

For the purposes of the present invention, "filaggrin unit" is understood to mean a polypeptide whose sequence is that of the product of translation of any one of the subunits encoding a filaggrin domain of the gene for human profilaggrin or from another species, or alternatively is a consensus sequence, a theoretical sequence obtained from the sequences of the filaggrin domains.

For the purposes of the present invention, "related molecule" is understood to mean any molecule having at least one arginine residue capable of being converted to a citrulline residue under the action of a PAD (peptidylarginine deiminase); by way of example, this PAD may be a rabbit muscle PAD, as shown in the examples below. It is however within the capability of persons skilled in the art to select any other appropriate PAD by simple routine tests, by reacting it with noncitrullinated human filaggrin.

The term "peptide" as used in the present application means in particular protein or protein fragment, oligopeptide, extracted, separated or substantially isolated or synthesized, especially those obtained by chemical synthesis or by expression in a recombinant organism; any peptide in whose sequence one or more amino acids of the L series are replaced by an amino acid of the D series, or vice versa; any peptide in which at least one of the CO-NH bonds, and advantageously all the CO-NH bonds of the peptide chain is (are) replaced with one or more NH-CO bonds; any peptide in which at least one of the CO-NH bonds and advantageously all the CO-NH bonds is or are replaced by one or more NH-CO bonds, the chirality of each aminoacyl residue, whether it is involved or not in one or more abovementioned CO-NH bonds, being either conserved or reversed in relation to the aminoacyl residues constituting a reference peptide, these compounds being also designated immunoretroids, a mimotope, and the like.

Antigens in accordance with the invention may for example be obtained by the action of PAD on natural, recombinant or synthetic peptides or proteins comprising arginine residues; they may also be obtained by peptide synthesis by directly incorporating one or more citrulline residues into the synthesized peptide.

According to a preferred embodiment of an antigen in accordance with the present invention, it consists of a polypeptide comprising all or part of the

sequence corresponding to amino acids 144 to 314 of a human filaggrin unit, in which at least one arginine residue has been replaced by a citrulline residue, or alternatively all or part of the sequence corresponding to amino acids 76 to 144 of a human filaggrin unit, in which at least one arginine residue has been replaced with a citrulline residue.

An antigen in accordance with the invention may for example consist of a peptide comprising all or part of the sequence corresponding to amino acids 71 to 119 or a human filaggrin unit, in which at least one arginine residue has been replaced with a citrulline residue.

Advantageously, an antigen in accordance with the invention consists of a peptide comprising all or part of at least one sequence derived from one of the sequences identified in the sequence listing in the annex under the numbers SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 6, by replacing at least one arginine residue with a citrulline residue.

The subject of the present invention is also the use of the antigens in accordance with the invention, as defined above, for the *in vitro* diagnosis of RA.

The present invention covers in particular antigenic compositions for diagnosing the presence of autoantibodies specific for RA in a biological sample, which compositions are characterized in that they contain at least one antigen in accordance with the invention, optionally labeled and/or conjugated with a carrier molecule.

The subject of the present invention is also a method of detecting class G autoantibodies specific for RA in a biological sample, which method is characterized in that it comprises:

- bringing said biological sample into contact with at least one antigen in accordance with the invention, as defined above, under conditions allowing the formation

of an antigen/antibody complex with the autoantibodies specific for RA which may be present;

- detecting, by any appropriate means, the antigen/antibody complex which may be formed.

This method of detection may be carried out using a kit comprising at least one antigen according to the invention, as well as buffers and reagents appropriate for constituting a reaction medium allowing the formation of an antigen/antibody complex, and/or means for detecting said antigen/antibody complex.

Said kit may also comprise, where appropriate, reference samples, such as one or more negative sera and one or more positive sera.

The present invention will be understood more clearly with the aid of the additional description which follows, which refers to examples of preparation and use of antigens in accordance with the invention.

EXAMPLE 1 : REACTIVITY OF SERA OBTAINED FROM PATIENTS SUFFERING FROM RHEUMATOID ARTHRITIS ON EPIDERMAL FILAGGRINS

A piece of human epidermis is ground with the aid of a "Potter" type electric grinder in a buffer with a high urea concentration (6 M), which makes it possible to solubilize all the epidermal filaggrins.

With this epidermal extract, two-dimensional electrophoresis (8-25% acrylamide gel in the presence of 6 M urea) is carried out; the 1st dimension corresponds to a gel isoelectrofocusing in a pH gradient ranging from 5 to 8 and the second dimension corresponds to electrophoresis under denaturing conditions, in the presence of SDS. After electrophoresis, the proteins in the gel are transferred onto nitrocellulose.

The immunological reactions are carried out according to a conventional protocol.

The nitrocellulose membrane is incubated overnight at 4°C with a serum from a patient suffering from RA, diluted 1/2000, and then the serum immunoglobulins which have reacted with the antigens

bound to the membrane are detected with the aid of a peroxidase-labeled anti-human IgG secondary antibody. The presence of the peroxidase substrate is revealed by the ECL (Enhanced ChemiLuminescence, AMERSHAM) method according to the protocol recommended by the manufacturer.

In a second stage, the same membrane is washed and then incubated for one and a half hours at 20°C in the presence, this time, of the monoclonal antibody AHF1 described by SIMON et al. [J. Invest. Dermatol. 105, 432, (1995)] at a concentration of 0.2 µg/ml, and then of a peroxidase-labeled anti-mouse IgG secondary antibody. The reaction is revealed by the ECL method, as indicated above.

The results are illustrated by Figure 1:

The monoclonal antibody AHF1 recognizes isoforms of filaggrin whose pHi ranges from 5.8 to 8.5. On the other hand, only the isoforms whose pHi ranges from 5.8 to 7.4 are detected by the serum from the patient suffering from RA.

The fact that only the most acidic isoforms of filaggrins are detected makes it possible to assume that the acidification of these isoforms forms part of the post-translational modifications which would be necessary for the recognition of filaggrin by the antibodies present in the sera from patients suffering from RA.

EXAMPLE 2 : *IN VITRO* DEIMINATION OF RECOMBINANT FILAGGRIN BY PEPTIDYLARGININE DEIMINASE (PAD)

Recombinant filaggrin is produced according to the following protocol:

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A DNA fragment encoding a filaggrin unit is amplified by PCR, using human genomic DNA (RAJI cells: ATCC CCL86) with the aid of the following 2 primers:

5' primer:

5' TTCCTATACCAGGTGAGCACTCAT 3'

3' primer:

5' AGACCCTGAACGTCCAGACCGTCCC 3'

The amplification product is cloned into the SmaI site of the vector pUC19. The recombinant clones are selected by verifying the presence of a 972 bp insert obtained after digestion with SacI and XbaI. This insert is then subcloned into pUC19. The insert resulting from this subcloning is then transferred into the vector pGEX (marketed by the company PHARMACIA), between the EcoRI and HindIII sites. The expression vector thus obtained expresses, in *E. coli*, filaggrin fused with glutathione S-transferase (GST), under the control of the prokaryotic Tac promoter. The synthesis of the recombinant protein is induced by addition of isopropyl-β-D-galactoside (IPTG) to the culture.

The recombinant filaggrin thus obtained will be called hereinafter: "fil-gst".

The existence of 9 fragments which result from post-translational proteolysis of the full-length filaggrin is observed after electrophoresis. The positions of the various cuts generating these fragments are indicated in Figure 2.

The mixture of the 9 fragments is subjected to deimination *in vitro* by peptidylarginine deiminase.

A rabbit muscle peptidylarginine deiminase preparation (681 U/ml) marketed by TAKARA BIOMED EUROPE is used according to the protocol recommended by the manufacturer.

The operating conditions are the following:

- reaction medium: 0.1 M Tris-HCl, 10 mM $CaCl_2$, 5 mM DTT, pH 7.4;
- enzyme/substrate ratio: 140 mU/μmol of filaggrin containing 10% arginine or 4 mU/μmol of arginine;
- incubation: between 0 and 60 min at 50°C;
- termination of the reaction: heating 3 min in LAEMMLI buffer.

The following 8 reactions are carried out in parallel.

(1) BSA (bovine serum albumin) incubated in reaction medium (1 h, 50°C) in PAD.

(2) BSA incubated in reaction medium (1 h, 50°C) with 60 mU of PAD.

(3) fil-gst incubated in reaction medium (1 h, 50°C) without PAD.

(4) fil-gst incubated in reaction medium (5 minutes at 50°C) with 60 mU of PAD.

(5) fil-gst incubated in reaction medium (15 minutes at 50°C) with 60 mU of PAD.

(6) fil-gst incubated in reaction medium (30 minutes at 50°C) with 60 mU of PAD.

(7) fil-gst incubated in reaction medium (1 h at 50°C) with 60 mU of PAD.

(8) fil-gst incubated in reaction medium (1 h at 50°C) with 60 mU of PAD and in the presence of 10 mM N-ethylmaleimide (PAD inhibitor).

1 µl of each sample is deposited on an electrophoresis gel (PHAST®-SDS gel 12.5%, PHARMACIA), and the electrophoresis is carried out with the PHAST-SYSTEM® apparatus (PHARMACIA), under the conditions recommended by the manufacturer. After transfer onto nitrocellulose, the revealing is carried out either with a pool of 5 sera from patients suffering from RA, diluted 1/2000 (Figure 3a), or with the anti-filaggrin monoclonal antibody AHF2 [SIMON et al. J. Invest. Dermatol. 105, 432, (1995)] at the concentration of 0.2 µg/ml (Figure 3b).

The antigen/antibody complex is revealed with the aid of a peroxidase-coupled secondary antibody by the ECL technique.

The results are illustrated by Figure 3

Lane 1 : BSA (1 hour, 50°C)
Lane 2 : BSA + PAD (1 hour, 50°C)
Lane 3 : fil-gst (1 hour, 50°C)
Lane 4 : fil-gst + PAD (5 minutes, 50°C)
Lane 5 : fil-gst + PAD (15 minutes, 50°C)
Lane 6 : fil-gst + PAD (30 minutes, 50°C)
Lane 7 : fil-gst + PAD (1 hour, 50°C)
Lane 8 : fil-gst + PAD + inhibitor (1 hour, 50°C)

In the absence of a citrullination reaction, the fil-gst is not recognized by the sera from patients suffering from RA (Figure 3a, lane 3), whereas from 5 minutes of citrullination (Figure 3a, lane 4), it is detected by these sera. An increase in reactivity is observed with the pool of sera when PAD is reacted for 60 minutes at 50°C (Figure 3a, lane 7).

- fragments 1, 2, 3 (bands identified by points) of the fil-gst are strongly recognized, after citrullination, by the sera from patients suffering from RA. Fragments 4 and 5 (bands identified by asterisks) are also recognized. These results make it possible to assume that one or more epitopes with a high affinity exist in the COOH-terminal half of filaggrin (144 to 314), this epitope being repeated between positions 76 and 144.

- the monoclonal antibody AHF2 recognizes all the fragments of fil-gst, citrullinated or otherwise.

EXAMPLE 3 : SPECIFICITY OF THE RECOGNITION OF CITRULLINATED FIL-GST BY THE SERA

In a first series of experiments (Figure 4a), the reactivity of noncitrullinated fil-gst (fil-gst alone, 30 minutes at 50°C) and fil-gst citrullinated with PAD 30 minutes at 50°C is compared with human sera composed of:

- sera from normal persons: T(2) and T(3)

- sera from patients suffering from RA having high AFA titers which are detected by immunotransfer on acido-neutral variants of human filaggrin, and by indirect immunofluorescence on cryosections of rat esophagus: RA(6) and RA(8);

- anti-filaggrin antibodies purified from the serum from a patient suffering from RA by affinity chromatography, on a column grafted with acido-neutral isoforms of human filaggrin: AFA.

A positive control is also carried out with the monoclonal antibody AHF2.

In a second series of experiments (Figure 4b), the reactivity of citrullinated fil-gst is confirmed with a larger series of sera:

- 4 control sera: T(4)
- 4 sera from patients suffering from RA not having AFAs detectable by immunotransfer or by indirect immunofluorescence: RA(4)
- 9 sera from patients suffering from RA with high AFA titers (three of them (*) were also tested in the first series of experiments): RA(9)
- anti-filaggrin antibodies purified by affinity chromatography, on a column grafted with the acido-neutral isoforms of filaggrin, from a pool of sera from 40 patients suffering from RA: AFA
- the monoclonal antibodies AHF (1-7).

The sera were used at the dilution of 1/2000; the anti-filaggrin antibodies purified by affinity chromatography are used at the concentration of 4 µg/ml; the monoclonal antibodies are used at the concentration of 0.2 µg/ml.

The results are the following:

- the citrullination of recombinant filaggrin is necessary for the recognition by the AFAs of the sera from patients suffering from RA (14 positive sera out of 14 recognize it);
- the antifilaggrin autoantibodies, purified by affinity chromatography from the sera from patients suffering from RA, show the same reactivity on citrullinated fil-gst as the sera from patients suffering from RA (recognition of the fragments corresponding to lanes 1 to 5). This shows that it is indeed the AFAs present in these sera which recognize the citrullinated fil-gst.

EXAMPLE 4 : CITRULLINATION OF THE PEPTIDES S-47-S AND S-35-R BY PAD, AND TEST OF THE REACTIVITY OF THE CITRULLINATED PEPTIDES.

The peptide of 49 amino acids S-47-S having the sequence (1-letter code):

NH_2-STGHSGSQHSHTTTQGRSDASRGSSGSRSTSRETRDQEQSGDGSRHSGS-COOH

corresponding to amino acids 71 to 119 of the sequence of a human filaggrin unit, and comprising 6 arginine residues, and

the peptide of 37 amino acids S-35-R having the sequence (1-letter code):

NH_2-SQDRDSQAQSEDSERRSASASRNHRGSAQEQSRDGSR-COOH

corresponding to amino acids 155 to 191 of the sequence of a human filaggrin unit, and comprising 7 arginine residues, were prepared by peptide synthesis. The peptides S-47-S and S-35-R are represented in the sequence listing in the annex under the respective numbers SEQ ID NO: 3 and SEQ ID NO: 4.

These 2 peptides, as well as fil-gst, were citrullinated by the action of PAD, for 30 minutes at 50°C, in the same reaction medium as that indicated in Example 2. The specific conditions for each peptide, and for the fil-gst are the following:

- peptide S-47-S: 4 mU/µmol arginine
- peptide S-35-R: 2.7 mU/µmol arginine
- fil-gst: as indicated in Example 2.

The reactivity of each peptide and that of fil-gst, before and after action of the enzyme, towards the monoclonal antibody AHF4, and the serum from a patient suffering from RA, is compared by dot-blot.

The operating conditions are the following:

- 0.5 µg by deposition of each antigen (peptides, fil-gst, acido-neutral variants of filaggrin (AVF))
- nitrocellulose treatment 45 minutes at 80°C, before immunodetection
- RA serum used at the dilution of 1/2000; monoclonal antibody AHF4 used at a concentration of 0.2 µg/ml

The results are illustrated by Figure 5, which shows that:

- AHF4 recognizes the peptide S-47-S and fil-gst, citrullinated or not, but does not recognize S-35-R, citrullinated or not.

- S-47-S is recognized, after citrullination, by the serum from the patient suffering from RA, whereas S-35-R, citrullinated or not, is not recognized. The same serum recognizes, moreover, the AVFs and the citrullinated fil-gst but does not recognize the noncitrullinated fil-gst.

EXAMPLE 5 : SYNTHESIS OF THE PEPTIDES E-12-H AND E-12-D CITRULLINATED AND NONCITRULLINATED AND TEST OF THE REACTIVITY OF THE PEPTIDES.

The peptides E-12-H and E-12-D were determined with reference to the nucleotide sequences of the gene for human profilaggrin which are described by GAN S.Q et al. [Biochemistry, 29: 9432-9440, (1990)].

The peptide of 14 amino acids E-12-H having the sequence (1-letter code):

NH_2-EQSADSSRHSGSGH-COOH

comprises 1 arginine residue, and

the peptide of 14 amino acids E-12-D having the sequence (1-letter code):

NH_2-ESSRDGSRHPRSHD-COOH

comprises 3 arginine residues.

The peptides E-12-H and E-12-D are represented in the sequence listing in the annex under the respective numbers SEQ ID NO: 5 and SEQ ID NO: 6.

These peptides were prepared by solid phase peptide synthesis.

The citrullinated peptides E-12-H and E-12-D were directly synthesized by incorporation of a citrulline by replacing an arginine.

For the peptide E-12-D, only the arginine residue corresponding to the 8th amino acid of the sequence was replaced by a citrulline during peptide synthesis.

The reactivity of each citrullinated and noncitrullinated peptide was tested respectively in relation to a normal serum, to two sera from RA patients, to anti-filaggrin antibodies (AFAs) purified from a pool of 45 sera from RA patients and to anti-

filaggrin antibodies purified from 12 sera from RA patients.

EXPERIMENTAL PROTOCOL:

The wells of NUNC MAXISORP microtiter plates were respectively coated with the aid of the noncitrullinated and citrullinated peptides E-12-D and E-12-H, diluted to a concentration of 5 µg/ml in a PBS buffer (pH: 7.4) and incubated overnight at 4°C (final volume: 100 µg/well). The wells were saturated for 30 minutes at 37°C in PBS-Tween 20, 0.05%, 2.5% gelatin, 200 µl/well. The negative control serum (normal serum) was diluted 1/120. The anti-filaggrin antibodies were diluted in PBS-Tween 20, 0.05% - 0.5% gelatin (PBS TG) such that the final anti-filaggrin autoantibody concentrations are those indicated in the accompanying Table I. The negative control serum, the RA sera and the anti-filaggrin antibodies were added (final volume: 100 µl/well) and incubated for 1 hour at 37°C and overnight at 4°C. Peroxidase-labeled goat antibodies anti-gamma heavy chains of the human immunoglobulins (marketed by the company SOUTHERN BIOTECHNOLOGIES) were added to each well (dilution in PBSTG: 1/2000, final volume: 100 µl/well) and incubated for 1 hour at 37°C. The revealing was carried out by addition of ortho-phenylenediamine (2 mg/ml, for 10 minutes).

The results presented in the accompanying Table I are given as a ratio of OD at 492 nm: citrullinated peptide signal/noncitrullinated peptide signal.

These results show that in the majority of cases, the citrullinated peptide/noncitrullinated peptide OD ratio is greater than 1, and therefore illustrate the good sensitivity of the citrullinated peptides compared with the non-citrullinated peptides for their reactivity toward the anti-filaggrin autoantibodies.

TABLE 1

Peptide	Control serum	RA1 serum		RA2 serum			Pool of AFAs			AFAs purified from 12 RA sera											
		10*	20*	5*	10*	20*	5*	10*	20*	10*	10*	10*	10*	10*	10*	10*	10*	10*	10*	10*	10*
E-12-D	1.076	1.42	1.85	2.42	3.77	5.57	1.77	1.63	1.48	1.99	1.38	2.48	1.19	1.12	3.50	1.87	5.19	1.13	1.57	1.11	1.65
E-12-H	1	1.32	1.20	10.44	11.51	8.38	2.45	2.42	1.82	7.16	2.05	1.06	1.18	0.76	13.57	4.14	3.18	1.14	3.66	1.22	5.84

*: Concentration of AFAs in μg/ml.